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Development and validation of UPLC-UV method for the determination of rubraxanthone in human plasma

[Desarrollo y validación del método UPLC-UV para la determinación de rubraxantona en plasma humano]

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Abstract

Context: Rubraxanthone has potential health benefits, such as antioxidant, anti-bacterial and cytotoxic agent. A sensitive method is required to quantify plasma concentrations to assess its efficacy.

Aims: To develop and validate an analytical method for the determination rubraxanthone in human plasma using ultra-performance liquid chromatography (UPLC-UV) for pharmacokinetic application.

Methods: Chromatographic separation was performed aced using a C18 column (100 mm × 3.0 mm, particle size 1.8 µm) with a mobile phase consisting of acetonitrile – 0.4% formic acid (75:25, v/v). The isocratic flow rate was 0.3 mL/min with elution time for rubraxanthone was approximately 3 min and UV detection were at 243 nm. Biological sample preparation involved protein precipitation method with acetonitrile. The developed method was validated as EMEA guidelines for its selectivity, linearity, sensitivity, precision, accuracy, recovery and stability.

Results: The method was proven to be linear over a plasma rubraxanthone concentration range of 206 to 6180 ng/mL with a mean correlation coefficient of 0.999. The within-run and between-run precision (coefficient of variation) were less than 4.7%. The mean recovery of rubraxanthone from human plasma was found to be greater than 95%. The lower limits of quantitation of the method was determined to be 206 ng/mL. The samples remained stable during the processing and analysis times and also during the three freeze/thaw cycles.

Conclusions: The UPLC-UV method was validated for all of the criteria that were necessary for a bioanalytical method and could reliably quantitate rubraxanthone in human plasma for future clinical pharmacokinetic study.

Keywords: bioanalysis; rubraxanthone; UPLC; validation.

Resumen

Contexto: Rubraxanthone tiene beneficios potenciales para la salud, como agente antioxidante, antibacteriano y citotóxico. Se requiere un método sensible para cuantificar las concentraciones plasmáticas para evaluar su eficacia.

Objetivos: Desarrollar y validar un método analítico para la determinación de rubraxantona en plasma humano utilizando cromatografía líquida de ultra alto rendimiento (UPLC-UV) para aplicación farmacocinética.

Métodos: La separación cromatográfica se realizó con una columna C18 (100 mm x 3,0 mm, tamaño de partícula 1,8 µm) con una fase móvil de acetonitrilo - 0,4% de ácido fórmico (75:25, v/v). La velocidad de flujo isocrático fue de 0,3 mL/min con un tiempo de elución para rubraxantona de aproximadamente 3 minutos y la detección UV fue a 243 nm. La preparación de la muestra biológica incluyó el método de precipitación de proteínas con acetonitrilo. El método desarrollado fue validado mediante pautas EMEA por su selectividad, linealidad, sensibilidad, precisión, exactitud, recuperación y estabilidad.

Resultados: Se demostró que el método fue lineal en un rango de concentración de rubraxantona en plasma de 206 a 6180 ng/mL con un coeficiente de correlación medio de 0,999. La precisión dentro del ciclo y entre ciclos (coeficiente de variación) fue inferior al 4,7%. Se encontró que la recuperación media de rubraxantona del plasma humano fue superior al 95%. Se determinó que los límites inferiores de cuantificación del método eran 206 ng/mL. Las muestras permanecieron estables durante los tiempos de procesamiento y análisis y también durante los tres ciclos de congelación/descongelación.

Conclusiones: El método UPLC-UV fue validado para todos los criterios necesarios para un método bioanalítico y podría cuantificar de manera confiable la rubraxantona en plasma humano para futuros estudios clínicos farmacocinéticos.

Palabras Clave: bioanálisis; rubraxantona; UPLC; validación.

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INTRODUCTION

Rubraxanthone (Fig. 1) is a pharmacologically active constituent isolated from stem bark of *Garcinia cowa* (Ritthiwigrom et al., 2013). Numerous studies indicate that rubraxanthone exerts a variety of bioactive and pharmacological effects, such as anti-inflammatory agent (Wahyuni et al., 2017), antimicrobial and antioxidant (Lee and Chan, 1977; Dachriyanus et al., 2003). It also has anticancer activity against breast cancer (MCF-7), human prostate cancer (DU-145) and lung cancer (H-460) (Wahyuni et al., 2015) as well as can reduce total cholesterol and triglyceride levels in the blood of male rats (Dachriyanus et al., 2006).

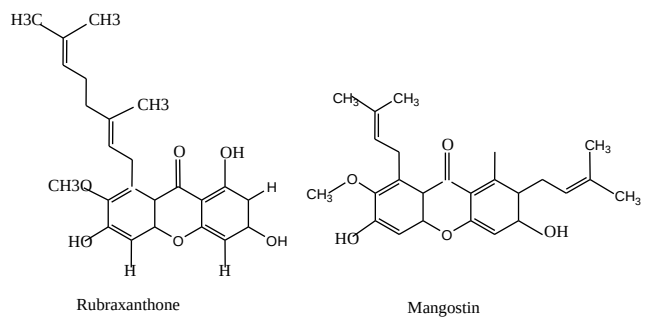

Figure 1. Chemical structure of rubraxanthone and mangostin (IS).

Several analytical methods for determining rubraxanthone in *G. cowa* bark extract using HPTLC (Dachriyanus et al., 2017) and HPLC (Susanti et al., 2018) were published. However, there is no method reported for the determination of rubraxanthone in human plasma. This study describes the simple, fast, precise, and accurate UPLC-UV method for determining rubraxanthone in human plasma.

MATERIAL AND METHODS

Instrumentation

UPLC-UV system (Agilent 1290 Infinity II LC system, Germany) equipped with G7102A high speed and G7117B photo Diode Array Detector (DAD) was utilized.

Chemicals and reagents

Rubraxanthone was isolated from *G. cowa* Roxb (Susanti et al., 2018). Mangostin as internal standard (IS) was purchased from Andalas Sitawa Fitolab. The structure for rubraxanthone, $C_{24}H_{26}O_6$ and mangostin are depicted in Fig. 1. Acetonitrile and methanol were HPLC-grade and were purchased from Merck. The other chemicals and reagents were analytical grade.

Chromatographic conditions

The analytical column ZORBAX RRHD Eclipse Plus C18 (100 mm × 3.0 mm id, 1.8 µm) was used. The mobile phase consisted of a mixture of acetonitrile and 0.4% formic acid, at volume ratio of 75:25. It was prefiltered through a Millipore 0.22 µm filter followed by sonication prior to use for analysis and pumped at a flow rate of 0.3 mL/min. The DAD was set in 243 nm. The column temperature was set at 20 ± 3°C.

Preparation of standard solutions and quality control samples

Primary stock solutions of rubraxanthone (1 mg/mL) and mangostin (1 mg/mL) were prepared in methanol. Then, diluted with methanol to obtain the certain concentration. Human plasma was obtained from Indonesian Red Cross Society. Human plasma calibration standards of rubraxanthone were prepared by spiking an appropriate amount of the working standard solutions into human plasma. The concentration range of rubraxanthone in calibration curve was 206–6180 ng/mL and Quality Control (QC) samples were prepared at three concentrations that were low (616 ng/mL), medium (2570 ng/mL), and high (4934 ng/mL).

Sample preparation

A volume of 150 µL of human plasma containing certain concentrations of rubraxanthone was added 50 µL of mangostin working solution (20 µg/mL) vortex-mixed for 30 sec. Three parts of

acetonitrile (300 µL) were added to the precipitate protein in plasma, vortex-mixed for 2 min and centrifuged at 10 000 rpm for 10 min. A 5 µL aliquot of the supernatant was injected into the UPLC-UV system.

Validation of method

The method was validated in accordance with EMEA (2011).

Selectivity

Selectivity was studied by comparing chromatograms of six blank plasma samples with plasma samples spiked with rubraxanthone and mangostin. Each blank sample was tested for interference, and selectivity was ensured at the lower limit of quantification (LLOQ). Absence of interfering components was accepted where the % diff was less than 20% of the lower limit of quantification for the analyte and 5% for the internal standard.

Linearity

Linearity of the analytes was evaluated using freshly prepared samples covering the analysis range. The calibration curve of rubraxanthone was constructed by plotting the peak area ratios of the respective analyte to the internal standard (y) against the analyte concentration (x) (expressed as ng/mL). The procedure was carried out in triplicate for each concentration. The lowest concentration of the analytes with an accuracy of 80–120%, as well as the coefficient of variation of <20% was regarded as the lowest limit of quantification (LLOQ). This concentration was used as the first calibration point for the calibration curve.

The applied requirements for a valid calibration model were a regression coefficient higher than 0.990 (R^2), and the residuals and coefficient of variation (CV%) to be within ±20% at the lower limit of quantification (LLOQ) and ± 5% for the rest of the concentrations tested.

Recovery

The extraction recovery of rubraxanthone was assessed by assaying three sets of samples at three concentration levels (616, 2570 and 4934 ng/mL) rubraxanthone and mangostin were: (A) spiked into plasma before extraction; and (B) spiked directly into equivalent volumes of acetonitrile. The extraction recovery, also known as absolute recovery, was determined by comparing the rubraxanthone peak area obtained from Set A samples with that from Set B samples.

Precision and accuracy

For precision and accuracy studies, samples were prepared at four concentration levels: the lower limit of quantification (LLOQ), low (LQC), medium (MQC), and high (HQC) quality controls, corresponding to 206, 616, 2570, and 4934 ng/mL of rubraxanthone , respectively, with six replicates each. The within-run and between-run precision were determined by analyzing the prepared samples on the same and three different days, respectively. Precision was characterized by the percent coefficient of variation (%CV) whereas accuracy was expressed %diff.

Carry over

During the validation process, carry-over was determined by injecting blanks after previously injected with upper limit of quantification of analyte (ULOQ) on five replicates. Carry over on blanks should not be more than 20% of LLOQ and 5% for default

Stability

The stability of rubraxanthone in human plasma was investigated by analyzing QC samples at LQC of (616 ng/mL) and HQC (4934 ng/mL) after storage under different conditions: (1) the freeze-thaw stability of rubraxanthone in human plasma was evaluated after three freeze-thaw cycles; (2) the short-term stability was determined by placing plasma samples at room temperature (25°C) for 6 h; (3) the long-term stability was tested after storing the samples at −20°C for 1 month or at 4°C for 15 days; (4) the post-preparative stability was evaluated after keeping the samples in the autosampler at 20°C for 24 h.

RESULTS

Optimization of sample preparation

An efficient sample preparation method is very important for accurate and reliable UPLC-UV assays. A simple and rapid protein precipitation pretreatment was employed in this study. Acetonitrile was chosen as the solvent for sample preparation, because it exhibited to be the superior organic plasma protein precipitant, particularly at volume ratios, 2:1 (precipitant: plasma).

Optimization method

The developed UPLC-UV method was applied for determination of rubraxanthone in human plasma. To optimize the UPLC-UV assay parameters, the mobile phase composition was studied. A satisfactory separation was obtained with a mobile phase consisting of acetonitrile – 0.4% formic acid (75:25, v/v) with a flow rate 0.3 mL/minute. Increasing acetonitrile concentration to more than 77% led earlier elution of the rubraxanthone peak but with excessive tailing. At lower acetonitrile concentration (<73%), separation occurred too late. The typical chromatograms for the blank plasma, rubraxanthone and mangostin (IS) are given in Fig. 2A-C. The system suitability parameters are given in Table 1.

Table 1. System suitability data.

Parameters	Value
Capacity factor (k')	3.517
Resolution factor (Rs)	8.666
Selectivity factor (α)	1.563
Number of theoretical plates (N)	13454

Method validation

Selectivity

Selectivity was evaluated by extracting six different blank plasma samples. The absence of interfering peaks at the retention time of rubraxanthone was considered as evidence for selectivity of the method. The chromatogram of rubraxanthone and mangostin standard solution in methanol is showed in Fig. 2A. This result presented 2 peaks with retention time 3.168 (rubraxanthone) and 4.551 (mangostin) respectively, with a resolution of 8.666. The chromatogram of blank plasma spiked with rubraxanthone and mangostin (Fig. 2C) also showed two peaks with retention time similar to chromatogram of rubraxanthone and mangostin standard solution while the chromatogram of blank plasma (Fig. 2B) showed no peak at retention time of rubraxanthone and mangostin. The result showed that there was no interference endogenous compound from the blank plasma of the six different sources.

Linearity and range (calibration curves)

The calibration curves of rubraxanthone in human plasma were obtained based on the peak area ratio of rubraxanthone to mangostin (y) *vs.* the rubraxanthone concentration (x), showing a good linear relationship over the range of 206 - 6180 ng/mL. A typical calibration curve equation was $y= 0.0004x + 0.0429$ ($R^2 = 0.9997$). The lower limit of quantification (LLOQ) of rubraxanthone in human plasma was 206 ng/mL; with the coefficient of variation (CV) of 2.84%, which was <20% (EMEA, 2011).

Recovery

The absolute recovery of rubraxanthone and mangostin is shown in Table 2. The extraction recovery for rubraxanthone ranged from 96.96 – 100.17% over the three QC concentration levels, while the extraction recovery of mangostin was 97.57%. These results suggested that the extraction method could provide high extraction efficiency (EMEA, 2011).

Precision and accuracy

Precision and accuracy were calculated by within-run and between-run variation of QC sample in six replicates at four concentrations as shown in Table 3. The precision (%CV) values of within-run and between-run analysis were 2.84 - 3.24% and 0.88 – 4.61%, respectively. Whereas the range for within-run accuracy (%different) for rubraxanthone was 0.27 – 8.91%, and range for between-run accuracy (%different) was 0.12 – 12.24%. These

results showed that this method fulfilled acceptance criteria of EMEA (2011) because these values were <15%.

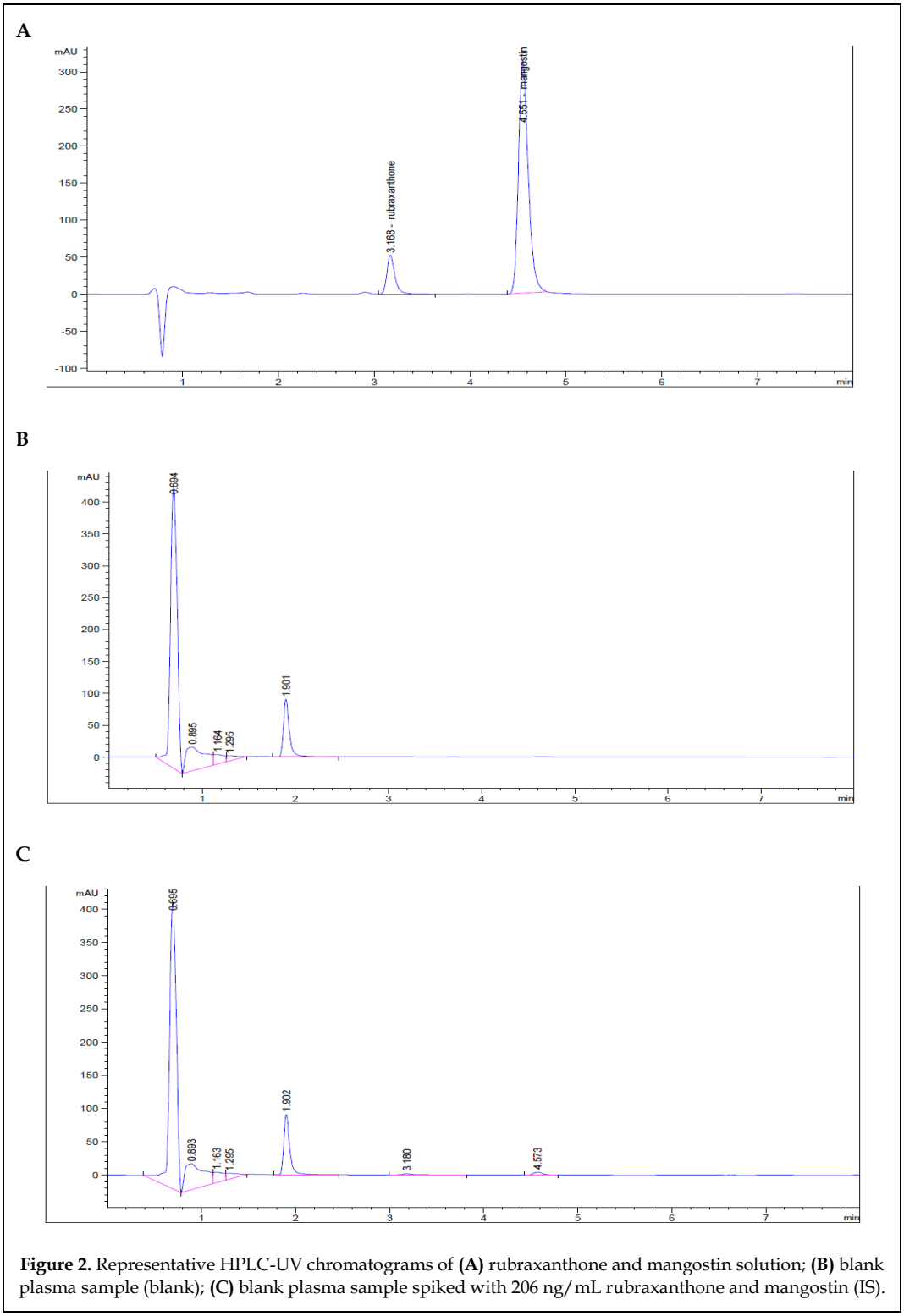

Figure 2. Representative HPLC-UV chromatograms of **(A)** rubraxanthone and mangostin solution; **(B)** blank plasma sample (blank); **(C)** blank plasma sample spiked with 206 ng/mL rubraxanthone and mangostin (IS).

Table 2. Recovery for rubraxanthone and mangostin in human plasma (n = 5).

Compound	Nominal concentration (ng/mL)	Recovery (mean ± SD)	% CV
Rubraxanthone	616	96.97 ± 0.78	0.80
	2570	100.17 ± 0.36	0.36
	4934	98.98 ± 1.23	1.24
Mangostin	2000	97.57 ± 2.68	2.75

Table 3. Accuracy and precision of rubraxanthone in human plasma.

Analyte	Actual concentration (ng/mL)	Mean measured concentration (ng/mL)	Precision (% CV)	Accuracy (% Diff)
Within-run	206	170.42 ± 4.84	2.84	14.24 - 19.54
	616	609.10 ± 21.21	3.24	0.93 - 8.91
	2570	2709.23 ± 79.07	2.92	0.27 - 8.15
	4934	4623.29 ± 84.88	2.84	5.96 - 8.48
Between-run	206	172.46 ± 3.24	3.01	12.32 - 16.01
	616	595.21 ± 12.23	4.61	1.16 - 12.24
	2570	2932 ± 43.56	0.88	0.14 - 2.54
	4934	4598 ± 67.45	1.36	6.12 - 9.23

Values are expressed as mean ± SD (n=6).

Carry over

Carry over after injection high concentration of rubraxanthone was in ranges from 2.02 - 3.37% of the LLOQ response, while the % carry-over the mangostin was in the range of 0.05 - 0.09%. Based on the research, the carry over test results did not exceed the requirements (should not be greater than 20% of the lower limit of quantification), so can be concluded during the analysis process takes place there was no carry over meaningful between injections.

Stability

The stability test of rubraxanthone in plasma was evaluated under different temperature and storage condition and was performed at LQC (616 ng/mL) and HQC (4934 ng/mL) in three replicates. The data for stability experiments are summarized in Table 4. These results suggested that rubraxanthone was stable under different storage conditions (stability data in Table 3).

DISCUSSION

During UPLC-UV method development, different options were examined to optimize detection parameters, chromatography conditions, and sample extraction. The UV detector was used for the estimation of rubraxanthone at 243 nm to maximize the signal of the drug and minimize the signal of plasma interferents. The compositions of mobile phase were optimized through several trials to achieve good resolution and symmetric peak shape for rubraxanthone. Optimization of UPLC-UV conditions performed on chromatographic parameters including; capacity factor, resolution and selectivity of the various compositions, and velocity of mobile phase. The mobile phase that resulted in optimum results was composed of a mixture of acetonitrile – 0.4% formic acid (75:25, v/v) with a flow rate 0.3 mL/minute. It was found that the values of the calculated parameters were within the acceptable limits at 243 nm, where the capacity factor (k') was between 2 - 10, resolution (R)

Table 4. Stability of rubraxanthone in human plasma under different storage conditions.

Storage condition	Actual concentration (ng/mL)	Mean measured concentration (ng/mL)	% Diff
3 Freeze thaw cycle	616	611.09 ± 11.03	0.49 - 2.89
	4934	4966.22 ± 59.27	5.94 - 10.65
Short-term stability for 6 h (25°C)	616	636.89 ± 23.72	0.63 - 7.06
	4934	4931.31 ± 49.02	4.16 - 8.02
Long-term stability for 1 month (−20°C)	616	582.82 ± 16.43	2.58 - 7.78
	4934	4893.25 ± 12.37	5.43 - 8.99
Long-term stability for 2 weeks (4°C)	616	601.31 ± 68.45	3.11 – 13.29
Autosampler for 24 h (20°C)	4934	4911.20 ± 57.78	6.90 - 10.65
	616	624.98 ± 31.27	0.49 – 7.06
	4934	4902.28 ± 5.65	4.16 – 5.94

Values are expressed as mean ± SD (n=3).

between the two eluted peaks was >2, selectivity factor (α) >1 and number of theoretical plates (N) > 2000.

An efficient sample preparation method is very important for accurate and reliable bioanalysis by UPLC-UV. A simple and rapid protein precipitation using acetonitrile pre-treatment employed in this work. Protein precipitation is commonly used for fast sample clean-up and disrupting protein–drug binding. Organic solvents are the most widely used protein precipitants utilized in drug analysis (Evans, 2004). Acetonitrile was found to be the superior organic plasma protein precipitant, particularly at volume ratios 2:1 (precipitant: plasma).

According to the International Conference on Harmonization (ICH-1995), the internal standard should be structurally similar to the main compound of analysis (ICH, 1995). Mangostin was selected as the internal standard due to the similarity of its structure to that of the rubraxanthone and its suitable chromatographic properties. This method also fulfils the acceptance criteria from EMEA (2011) for bioanalytical method validation.

CONCLUSIONS

The UPLC-UV method was valid for determination of rubraxanthone in human plasma and showed good chromatographic parameters including selectivity, linearity, sensitivity, accuracy, precision, stability and %recovery of extraction. The developed method also can be used to analyze the concentration of rubraxanthone for bioavailability and pharmacokinetics study. The pharmacokinetic studies of rubraxanthone will provide helpful information for rubraxanthone developments and applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Susanti M	Harahap Y	Itam A	Dachriyanus
Concepts or ideas	x			x
Design	x	x	x	x
Definition of intellectual content	x			x
Literature search	x			x
Experimental studies	x			
Data acquisition	x			
Data analysis	x	x	x	x
Statistical analysis	x	x	x	x
Manuscript preparation	x	x	x	x
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

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